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**September 28, 2004**

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**Certifying Officer**

# PROVISIONAL APPLICATION COVER SHEET

To the Commissioner of Patents and Trademarks
Washington, DC 20231
This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

| Docket No. | 14450PRO | Type a plus sign (+) inside this box → | + |
|---|---|---|---|



INVENTOR(s)/APPLICANT(s)

| Inventor | Addresses: |
|---|---|
| 1. Niefang YU | Singapore, Singapore (Chinese Citizen) |

TITLE OF THE INVENTION (280 characters max)

BENZIMIDAZOLE DERIVATIVES: PREPARATION AND PHARMACEUTICAL APPLICATIONS

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification Number of Pages 25

METHOD OF PAYMENT (check one)

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| ☒ A check or money order is enclosed to cover the<br>☒ The Commissioner is hereby authorized to charge any additional fees and credit any overages to Deposit Account Number: 04-1577 | Provisional filing fee amount(s) | $ 160.00 |

Respectfully submitted,

Signature: _____ Date: September 22, 2003

Typed or Printed Name: Ralph A. Dowell Registration No.: 26,868

# Benzimidazole derivatives: preparation and pharmaceutical applications

## FIELD

Embodiments are disclosed of hydroxamate compounds which are inhibitors of histone deacetylase. More particularly, there are disclosed certain benzimidazole containing compounds and methods for their preparation. These compounds may be useful as medicaments for the treatment of proliferative diseases.

## BACKGROUND

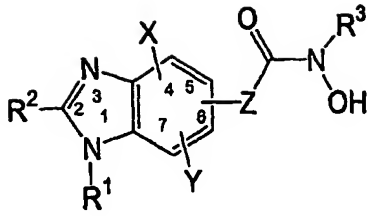
Local chromatin architecture is generally recognized as an important factor in the regulation of gene expression. The architecture of chromatin, a protein-DNA complex, is strongly influenced by post-translational modifications of the histones which are the protein components. Reversible acetylation of histones is a key component in the regulation of gene expression by altering the accessibility of transcription factors to DNA. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression [1,2]. In normal cells, histone deacetylase (HDACs) and histone acetyltransferase together control the level of acetylation of histones to maintain a balance. Inhibition of HDACs results in the accumulation of hyperacetylated histones, which results in a variety of cellular responses, such as apoptosis, necrosis, differentiation, inhibition of proliferation and cytostasis.

Inhibitors of HDAC have been studied for their therapeutic effects on cancer cells. For example, suberoylanilide hydroxamic acid (SAHA) is a potent inducer of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines [3,4]. SAHA has been shown to suppresses the growth of prostate cancer cells *in vitro* and *in vivo* [5]. Other inhibitors of HDAC that have been widely studied for their anti-cancer activities are trichostatin A (TSA) and trapoxin B [6,7]. Trichostatin A is a reversible inhibitor of mammalian HDAC. Trapoxin B is a cyclic tetrapeptide, which is an irreversible inhibitor of mammalian HDAC. However, due to the in vivo instability of these compounds they are less desirable as anti-cancer drugs. Recently, other small molecule HDAC inhibitors have become available for clinical evaluation [8]. Additional HDAC inhibiting compounds have been reported in the literature [Bouchain G. et al, J. Med. Chem., 46, 820-830 (2003)] and patents [WO 03/066579A2]. HDAC inhibitors have been reported to interfere with neurodegenerative processes, for instance, HDAC inhibitors arrest polyglutamine-dependent neurodegeneration [Nature, 413, 18 October, 2001].

Despite recent advances, there remains a need for an active compound with desirable activity, solubility, and metabolic properties that is suitable for treating cancerous tumors.

## SUMMARY

There are disclosed histone deacetylase inhibitor compounds that may be useful as pharmaceutical agents having the formula (I):



Formula I

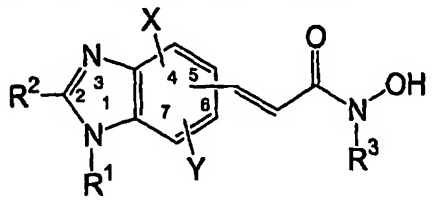
wherein

- $R_1$ is selected from hydrido, $C_1$ -$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_2$ is H, halo, or is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_3$ is selected from H, $C_1$ -$C_6$ alkyl, acyl;
- X and Y are the same or different and independently selected from hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6R_7$;
- R4 is selected from C1-C4 alkyl;
- R5 is selected from C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and independently selected from hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

- Z is a single bond or is selected from CH2, -CH2CH2-, CH=CH, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C1-C4 alkyl;

or a pharmaceutically acceptable salt thereof.

One suitable genus of hydroxamate compounds are those of formula Ia:



Formula Ia

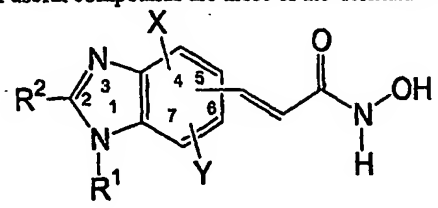
wherein

- $R_1$ is selected from hydrido, $C_1$ -$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_2$ is H, halo, or is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;

- $R_3$ is selected from H, $C_1$ -$C_6$ alkyl;, acyl;
- X and Y are the same or different and independently selected from hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6R_7$;
- R4 is selected from C1-C4 alkyl;
- R5 is selected from C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and independently selected from hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

Or a pharmaceutically acceptable salt thereof.

Another group of useful compounds are those of the formula Ib :



Formula Ib

wherein

- $R_1$ is selected from hydrido, $C_1$ -$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen, =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_2$ is H, halo, or is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- X and Y are the same or different and independently selected from hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6R_7$ ;
- R4 is selected from C1-C4 alkyl;
- R5 is selected from C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and independently selected from hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

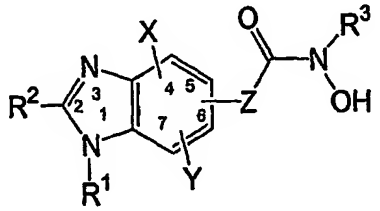
In addition to compounds of Formula I, the embodiments disclosed are also directed to pharmaceutically acceptable salts, pharmaceutically acceptable prodrugs, and pharmaceutically active metabolites of such compounds, and pharmaceutically acceptable salts of such metabolites. Such compounds, salts, prodrugs and metabolites are at times collectively referred to herein as "HDAC inhibiting agents".

The embodiments disclosed also relate to pharmaceutical compositions each comprising a therapeutically

effective amount of a HDAC inhibiting agent of the embodiments described with a pharmaceutically acceptable carrier or diluent for treating cellular proliferative ailments. The term effective amount as used herein indicates an amount necessary to administer to a host to achieve a therapeutic result, e.g., inhibition of proliferation of malignant cancer cells, benign tumor cells or other proliferative cells.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

There are disclosed hydroxamate compounds, for example benzimidazoles containing hydroxamic acid in one of the substituents, that may be inhibitors of deacetylases including but not limited to inhibitors of histone deacetylases [2]. The hydroxamate compounds may be suitable for treating tumors, including cancerous tumors. The hydroxamate compounds of the present embodiments have the following structure (I):




Formula I

wherein

- $R_1$ is selected from hydrido, $C_1$ -$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_2$ is H, halo, or is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;

- $R_3$ is selected from H, $C_1$ -$C_6$ alkyl, acyl;
- X and Y are the same or different and independently selected from hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6R_7$;
- R4 is selected from C1-C4 alkyl;
- R5 is selected from C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and independently selected from hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;
- Z is a single bond or is selected from CH2, -CH2CH2-, CH=CH, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C1-C4 alkyl;

or a pharmaceutically acceptable salt thereof.

As used herein, the term unsubstituted means that there is no substituent or that the only substituents are hydrogen.

The term "Halogen" represents chlorine, fluorine, bromine or iodine. The term "halo" represents fluoro, chloro, bromo and iodo.

The term "Alkyl" refers to a straight or branched $C_1$ -$C_6$ alkyl, unless otherwise noted. Examples of suitable straight and branched $C_1$ -$C_6$ alkyl substituents include methyl, ethyl, n-propyl, 2-propyl, n-butyl, sec-butyl, t-butyl, hexyl, and the like.

The term "Acyl" denotes a radical provided by the residue after removal of hydroxyl from an organic acid, examples of such radical being acetyl and benzoyl.

The term "Cycloalkyl" refers to a saturated or partially saturated, monocyclic or fused or spiro polycyclic, carbocycle from $C_3$ -$C_9$ per ring, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and the like, unless otherwise specified.

The above discussion of alkyl and cycloalkyl substituents also applies to the alkyl portions of other substituents, such as without limitation, alkoxy, alkyl amines, alkyl ketones, arylalkyl, heteroarylalkyl, alkylsulfonyl and alkyl ester substituents and the like.

The term "Heterocycloalkyl" refers to a 3 to 9 membered aliphatic rings, such as 4 to 7 membered aliphatic rings, containing from one to three heteroatoms selected from nitrogen, sulfur, oxygen. Examples of suitable heterocycloalkyl substituents include pyrrolidyl, tetrahydrofuryl, tetrahydrothiofuranyl, piperidyl, piperazyl, tetrahydropyranyl, morphilino, 1,3-diazapane, 1,4-diazapane, 1,4-oxazepane, and 1,4-oxathiapane.

The term "Heteroalkyl" refers to a straight- or branched-chain alkyl group having from 2 to 12 atoms in the chain, one or more of which is a heteroatom selected from S, O, and N. Exemplary heteroalkyls include alkyl ethers, secondary and tertiary alkyl amines, alkyl sulfides, and the like.

The term "Aryl" refers to a monocyclic, or fused polycyclic, aromatic carbocycle (ring structure having ring atoms that are all carbon) having from 5 to 12 atoms per ring. Examples of aryl groups include phenyl, naphthyl, and the like.

The term "Heteroaryl" refers to a monocyclic, or fused polycyclic, aromatic heterocycle (ring structure having a 5 to 7 member aromatic ring containing one or more heteroatoms selected from N, O and S). Typical heteroaryl substituents include furyl, thienyl, pyrrole, pyrazole, triazole, thiazole, oxazole, pyridine, pyrimidine, isoxazolyl, pyrazine, indole, benzimidazole, and the like.

In Formula I, as well as in Formulae Ia-Ib defining sub-sets of compounds within Formula I, there is shown a benzimidazole ring system. Within this ring system, there are substitutable positions at the –4-,5-, 6-, and 7-ring positions. In each of FormulaeI, Ia, and Ib, there is a requirement for attachment of an acidic moiety at one of the ring positions. This acidic moiety may be provided by but is not limited to groups containinga hydroxamic acid or salt derivatives of such acid which when hydrolyzed would provide the acidic moiety. In some embodiments the acidic moiety may be attached to the ring position through an alkylene group such as –CH2- or –CH2CH2-, or an alkenyl group such as –CH=CH-. Possible positions for attachment of the acidic moiety are the 5– and 6–ring positions.

It is understood that included in the family of compounds of Formula I are isomeric forms including diastereoisomers, enantiomers, tautomers, and geometrical isomers in "E" or "Z" configurational isomer or a mixture of E and Z isomers. It is also understood that some isomeric forms such as diastereomers, enantiomers, and geometrical isomers can be separated by physical and/or chemical methods and by those skilled in the art.

Some of the compounds of the disclosed embodiments may exist as single stereoisomers, racemates, and/or mixtures of enantiomers and /or diastereomers. All such single stereoisomers, racemates and mixtures thereof are intended to be within the scope of the ssubject matter described and claimed.

Additionally, Formula I is intended to cover, where applicable, solvated as well as unsolvated forms of the compounds. Thus, each formula includes compounds having the indicated structure, including the hydrated as well as the non-hydrated forms.

In addition to compounds of the Formula I, the HDAC inhibiting agents of the various embodiments include pharmaceutically acceptable salts, prodrugs, and active metabolites of such compounds, and pharmaceutically acceptable salts of such metabolites.

Pharmaceutically acceptable salts include, when appropriate, pharmaceutically acceptable base addition salts and acid addition salts, for example, metal salts, such as alkali and alkaline earth metal salts, ammonium salts, organic amine addition salts, and amino acid addition salts, and sulfonate salts. Acid addition salts include inorganic acid addition salts such as hydrochloride, sulfate and phosphate, and organic acid addition salts such as alkyl sulfonate, arylsulfonate, acetate, maleate, fumarate, tartrate, citrate and lactate. Examples of metal salts are alkali metal salts, such as lithium salt, sodium salt and potassium salt, alkaline earth metal salts such as magnesium salt and calcium salt, aluminum salt, and zinc salt. Examples of ammonium salts are ammonium salt and tetramethylammonium salt. Examples of organic amine addition salts are salts with morpholine and piperidine. Examples of amino acid addition salts are salts with glycine, phenylalanine, glutamic acid and lysine. Sulfonate salts include mesylate, tosylate and benzene sulfonic acid salts.

Preferred HDAC inhibiting agents include those having an IC50 value of 1 uM or less.

The hydroxamate compound, or salt thereof, is suitable for preparing pharmaceutical compositions, especially pharmaceutical compositions having deacetylase, especially histone deacetylase, inhibiting properties. The subject matter disclosed herein further includes pharmaceutical compositions comprising a pharmaceutically effective amount of one or more of the above-described compounds as active ingredient. Pharmaceutical compositions according to the present disclosure are suitable for enteral, such as oral or rectal, and parenteral administration to mammals, including man, for the treatment of tumors, alone or in combination with one or more pharmaceutically acceptable carriers.

The hydroxamate compound is useful in the manufacture of pharmaceutical compositions having an effective amount the compound in conjunction or admixture with excipients or carriers suitable for either enteral or parenteral application. Preferred are tablets and gelatin capsules comprising the active ingredient together with (a) diluents; (b) lubricants, (c) binders (tablets); if desired, (d) disintegrants; and/or (e) absorbents, colorants, flavors and sweeteners. Injectable compositions are preferably aqueous isotonic solutions or suspensions, and suppositories are advantageously prepared from fatty emulsions or suspensions. The compositions may be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure and/or buffers. In addition, the compositions may also contain other therapeutically valuable substances. The compositions are prepared according to conventional mixing, granulating or coating methods, respectively, and contain preferably about 1 to 50% of the active ingredient.

Suitable formulations also include formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

As discussed above, the compounds of the of the embodiments disclosed are useful for treating proliferative diseases. A proliferative disease is mainly a tumor disease (or cancer) (and/or any metastases). The inventive compounds are particularly useful for treating a tumor which is a breast cancer, genitourinary cancer, lung cancer, gastrointestinal cancer, epidermoid cancer, melanoma, ovarian cancer, pancreas cancer, neuroblastoma, head and/or neck cancer or bladder cancer, or in a broader sense renal, brain or gastric cancer; in particular (i) a breast tumor; an epidermoid tumor, such as an epidermoid head and/or neck tumor or a mouth tumor; a lung tumor, for example a small cell or non-small cell lung tumor; a gastrointestinal tumor, for example, a colorectal tumor; or a genitourinary tumor, for example, a prostate tumor (especially a hormone-refractory prostate tumor); or (ii) a proliferative disease that is refractory to

the treatment with other chemotherapeutics; or (iii) a tumor that is refractory to treatment with other chemotherapeutics due to multidrug resistance.

In a broader sense of the embodiments set out herein , a proliferative disease may furthermore be a hyperproliferative condition such as leukemias, hyperplasias, fibrosis (especially pulmonary, but also other types of fibrosis, such as renal fibrosis), angiogenesis, psoriasis, atherosclerosis and smooth muscle proliferation in the blood vessels, such as stenosis or restenosis following angioplasty.

Where a tumor, a tumor disease, a carcinoma or a cancer are mentioned, also metastasis in the original ,organ or tissue and/or in any other location are implied alternatively or in addition, whatever the location of the tumor and/or metastasis.

The compound is selectively toxic or more toxic to rapidly propiferating cells than to normal cells, particularly in human cancer cells, e.g., cancerous tumors, the compound has significant antiproliferative effects and promotes differentiation, e.g., cell cycle arrest and apoptosis. In addition, the hydroxamate compound induces p21, cyclin-CDK interacting protein, which induces either apoptosis or G1 arrest in a variety of cell lines.

Additionally compounds of the various embodiments disclosed herein may be useful for treating neurodegenerative diseases, and inflammation.

## SYNTHESIS OF DEACETYLASE INHIBITORS

The agents of the various embodiments may be prepared using the reaction routes and synthesis schemes as described below, employing the techniques available in the art using starting materials that are readily available. The preparation of particular embodiments is described in detail in the following examples, but the artisan will recognize that the chemical reactions described may be readily adapted to prepare a number of other agents of the various embodiments . For example, the synthesis of non-exemplified compounds may be successfully performed by modifications apparent to those skilled in the art, e.g., by appropriately protecting interfering groups, by changing to other suitable reagents known in the art, or by making routine modifications of reaction conditions. Alternatively, other reactions disclosed herein or known in the art will be recognized as having applicability for preparing other compounds of the various embodiments.

Reagents useful for synthesizing compounds may be obtained or prepared according to techniques known in the art.

In the examples described below, unless otherwise indicated, all temperatures in the following description are in degrees Celsius and all parts and percentages are by weight, unless indicated otherwise. Various starting materials and other reagents were purchased from commercial suppliers, such as Aldrich Chemical Company or Lancaster Synthesis Ltd., and used without further purification, unless otherwise indicated. Tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) were purchased from Aldrich in SureSeal bottles and used as received. All solvents were purified by using standard methods in the art, unless otherwise indicated.

The reactions set forth below were performed under a positive pressure of nitrogen, argon or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, and the reaction flasks are fitted with rubber septa for the introduction of substrates and reagents via syringe. Glassware was oven-dried and/or heat-dried. Analytical thin-layer chromatography was performed on glass-backed silica gel 60 F 254 plates (E Merck (0.25 mm)) and eluted with the appropriate solven ratios (v/v). The reactions were assayed by TLC and terminated as judged by the consumption of starting material.

The TLC plates were visualized by UV absorption or with a p-anisaldehyde spray reagent or a phosphomolybdic acid reagent (Aldrich Chemical, 20wt% in ethanol) which was activated with heat, or by staining in iodine chamber. Work-ups were typically done by doubling the reaction volume with the reaction solvent or extraction solvent and then washing with the indicated aqueous solutions using 25% by volume of the extraction volume (unless otherwise indicated). Product solutions were dried over anhydrous sodium sulfate prior to filtration, and evaporation of the solvents was under reduced pressure on a rotary evaporator and noted as solvents removed in vacuo. Flash column chromatography [Still et al, J. Org. Chem., 43, 2923 (1978)] was conducted using E Merck-grade flash silica gel (47-61 mm) and a silica gel:crude material ratio of about 20:1 to 50:1, unless otherwise stated. Hydrogenolysiss was done at the pressure indicated or at ambient pressure.

1H NMR spectra was recorded on a Bruker instrument operating at 400 MHz, and 13C-NMR spectra was recorded operating at 100 MHz. NMR spectra are obtained as CDCl3 solutions (reported in ppm), using chloroform as the reference standard (7.25 ppm and 77.00 ppm) or CD3OD (3.4 and 4.8 ppm and 49.3 ppm), or an internal tetramethylsilane standard (0.00 ppm) when appropriate. Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets. Coupling constants, when given, are reported in Hertz.

Mass spectra were obtained using LC/MS either in ESI or APCI. All melting points are uncorrected.

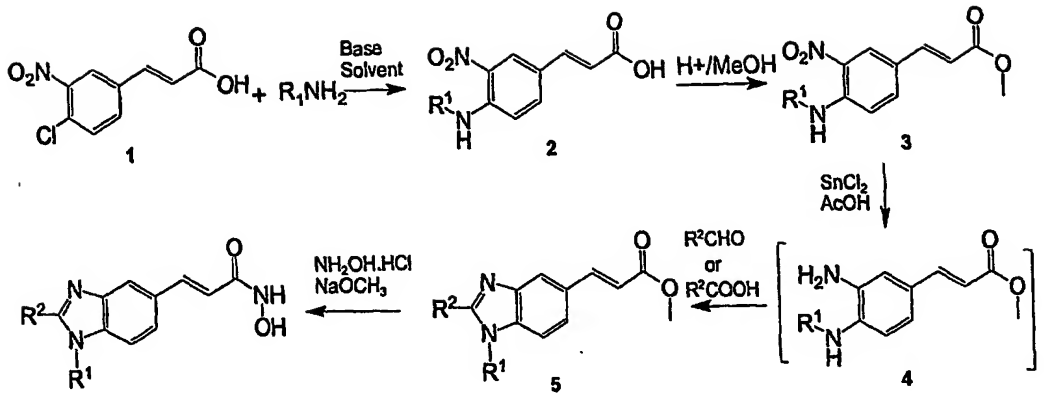
All final products had greater than 90% purity (by HPLC at wavelengths of 220 nm and 254 nm).

The following examples are intended to illustrate the embodiments disclosed and are not to be construed as being limitations thereto. Additional compounds, other than those described below, may be prepared using the following described reaction scheme or appropriate variations or modifications thereof.

## SYNTHESIS

Scheme I illustrates the procedure used for preparing compounds of formula Ib, wherein X and Y are hydrogens. Compounds of formula I can be prepared by analogous procedure, for example, by the choice of appropriate starting material. For example, in the case of Z is –CH2- in Formula I, such compound(s) can be synthesized by analogous method illustrated in Scheme I starting with a substituted phenylacetic acids (e.g.3-nitro-4-chlorophenyacetic acid), appropriate amine component (R1NH2), aldehyde or carboxylic acid component (R2CHO or R2COOH), and appropriate hydroxylamine or N-alkyl hydroxylamine (NHR3OH where R3 is defined as above).

Scheme I




Formula Ib

Specifically, the hydroxamate compounds Formula Ib can be synthesized by the synthetic route shown in Scheme 1. The reaction of *trans*-4-chloro-3-nitrocinnamic acid (1) with an amine in the present of a base (e.g., triethylamine) in an appropriate solvent (e.g., dioxane) gave (2). Treatment of (2) in methanol under acid catalysis (e.g., sulfuric acid) resulted in esterification providing (3). The nitro group of (3) can be reduced by appropriate reducing agent (e.g., Tin Chloride) and the resulting phenylenediamine was cyclized with an aldehyde to give (5). The hydroxamate compounds were obtained by a known synthesis method (J. Med. Chem., 2002, *45*, 753-757). An alternative method for preparation of (5) is by coupling (4) with an appropriate acid and then cyclized by heating with acetic acid (J. Med. Chem. 2001, *44*, 1516-1529).

The following preparation and examples are given to enable those skilled in the art to more clearly understand and to practice the subject matter hereof. They should not be considered as limiting the scope of the disclosure, but merely as being illustrative and representative thereof.

**Example 1**

Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylamide

Step 1

To a pre-stirred solution of *trans*-4-chloro-3-nitrocinnamic acid (1.0g, 4.4 mmol) in dioxane (10ml) was added triethylamine (2ml), 3-amino-1-propanol (1.5 ml). The resulting solution was heated to 85°C for 19 hours and then cooled to room temperature. The solvent was removed under vacuum. Water (100 ml) was added to the residue and the pH was adjusted to 1-1.5. The precipitate was collected and washed with cold water for 2 times and dried. The product 3-[3-nitro-4-(hydroxypropylamine)-phenyl]-acrylic acid was obtained as yellow solid (1.10g, 95%). MS(m/z): 267 $(MH)^+$

Step 2

Concentrated sulfuric acid (0.5 ml) was added to the solution of *trans*-4-(3-hydroxypropylamine)-3-nitrocinnamic acid, (1.10g, 3.9 mmol) and MeOH (15ml). The resulting solution was heated to reflux for 18 hours. The reaction mixture was cooled at –10° to -15°C for 3 hours. 3-[3-nitro-4-(hydroxypropylamine)-phenyl]-acrylic acid methyl ester was collected as crystalline yellow solid (1.06g, 91%). MS(m/z): 281 $(MH)^+$

Step 3

To a pre-stirred solution of methyl trans-4-(3-hydroxypropylamine)-3-nitrocinnamate (280 mg, 1.0 mmole) and 3-phenylbutyraldehyde (500mg, 3.4 mmole) in glacial acetic acid (5ml), Tin chloride was added (1.18g, 10.0 mmoles). The resulting solution was heated to 45°C for 17 hours and then cooled to room temperature. The solvent was removed under vacuum. Water (20 ml) and dichloromethane (20 ml) was added to the residue and stirred for 30 minutes. The organic layer was dried (MgSO4), filtered and concentrated to an oily residue. 100 ml diethyl ether was added and stirred for 4 hours. The product 3-[1-(3-Hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylic acid methyl ester was obtained in 34.9% yield (132.0 mg). MS(m/z): 379 $(MH)^+$

Step 4

Sodium methoxide (30% in methanol) (782mg, 4.1 mmole) was added to a prestirred solution of 3-[1-(3-Hydroxy-propyl)-2-(2-phenyl-propyl)-1H-benzimidazol-5-yl]-acrylic acid methyl ester (130mg, 0.34 mmole) and hydroxylamine hydrochloride (242 mg, 3.4 mmole) in MeOH (1.5 ml). The reaction mixture was continuously stirred for 40 minutes at room temperature and then poured into a solution of ice-water containing 1.0 ml concentrated hydrochloric acid. The mixture was extracted with dichloromethane. The organic layer was dried (MgSO4), filtered and concentrated. The desired product was separated by reverse phase preparative HPLC. After lyopholyzation, 7.8 mg (6%) of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylamide was obtained as powder. HPLC: 96%; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5µ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.22 min; 92%. $^1$H NMR (400 MHz, DMSO-$d_6$, δ): 1.35 (3H, d, J=6.5Hz), 1.83 (2H, m), 3.00-4.00 (6H, m), 4.33 (2H, t, J=7.1 Hz), 6.55 (1H, d, J=15.8Hz), 7.19-7.33 (5H, m), 7.62 (1H, d, J=15.8Hz), 7.70 (1H, d, J=8.60), 7.82 (1H, d, J=8.60Hz), 7.92 (1H, s), 10.15 (1H, bs), 10.33 (1H, bs). MS(m/z): 380 $[MH]^+$.

**Example 2**

Preparation of *N*-Hydroxy-3-[1-(3,4,5-trimethoxybenzyl)-2-(2-phenyl-ethyl)-1H-benzimidazol-5-yl]-acrylamide.

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 91%; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5µ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.22 min. $^1$H NMR (400 MHz , DMSO-$d_6$, δ): 3.08 (2H, t, J=7.72Hz), 3.48 (2H, t, 7.72Hz), 3.63 (3H, s), 3.67 (6H, s), 5.58 (2H, s), 6.59 (2H, s), 7.22-7.31 (7H, m), 7.63 (1H, d, J=15.78Hz), 7.71 (1H, d, J=8.76Hz), 7.83 (1H, d, J=8.76Hz), 7.98 (1H, s), 11.00 (2H, bs). MS(m/z): 488 $[MH]^+$.

**Example 3**

Prepartion of N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-methyl-1*H*-benzimidazole-5-yl]-acrylamide.

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 92%; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5µ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.32 min. . $^1$H NMR (400 MHz , DMSO-$d_6$, δ): 3.87 (3H, s), 4.01 (3H, s), 5.24 (2H, s), 6.56 (1H, d=15.80Hz), 7.32-7.50 (8H, m), 7.74(1H, d, J=8.72Hz), 7.88(1H, d, J=8.72Hz), 7.94(1H, s), 10.85(1H, bs). ). MS (m/z): 431 $[MH]^+$.

**Example 4**

Preparation of N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-(3-hydroxy-propyl)-1*H*-benzimidazole-5-yl]-acrylamide.

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 95%; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5µ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 6.82 min. . $^1$H NMR (400 MHz , DMSO-$d_6$, δ): 1.96 (2H, m), 3.88(3H, s), 4.48 (2H, t, J=7.12Hz), 5.24 (2H, s), 6.56 (1H, d, J=15.76Hz), 7.32-7.50(8H, m), 7.65 (1H, d, J=15.76Hz), 7.74 (1H, d, J=8.60Hz), 7.91 (1H, d, J=8.60Hz), 7.95 (1H, s), 10.85(1H, bs). MS (m/z): 474 $[MH]^+$.

**Example 5**

Preparation of N-Hydroxy-3-[1-(2-hydroxy-ethyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide.

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98%; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5µ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile

with 0.1% trifluoroacetic acid; UV 254): 4.12 min. $^1$H NMR (400 MHz , DMSO-$d_6$, δ): 3.80(2H, t, J=5.36Hz), 3.87 (3H, s), 4.39(2H, t, J=5.36Hz), 6.56(1H, d, 15.72Hz), 7.17 (2H, d, J=8.88Hz), 7.61(1H, d, J=8.52Hz), 7.62(1H, d, J=15.72Hz), 7.78(1H, d, J=8.52Hz), 7.88(1H, d, J=8.88Hz), 7.90(1H, s), 10.77(1H, bs). MS (m/z): 354 $[MH]^+$.

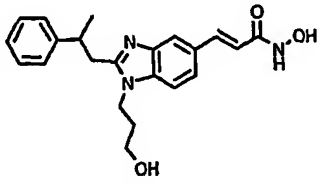
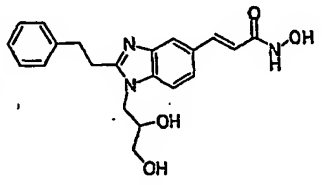
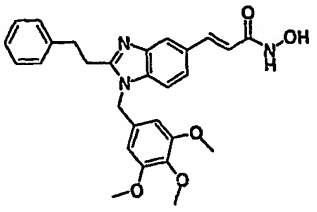
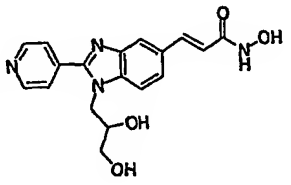
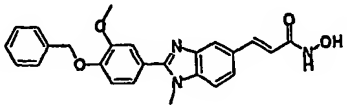
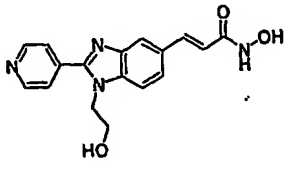
**Example 6**

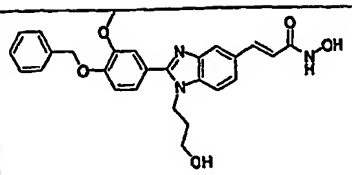
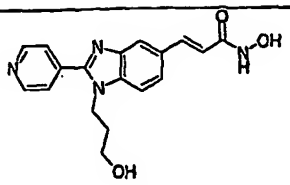
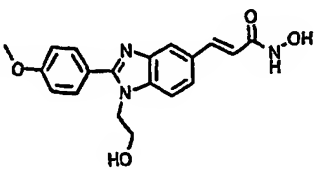
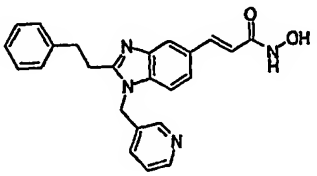
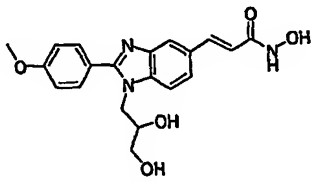
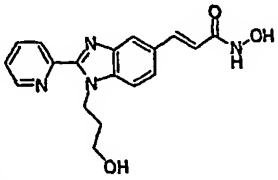
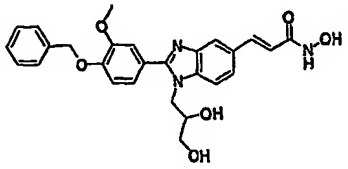
Preparation of N-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide.

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98%, min; $t_R$=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: $H_2O$ with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.389 min. NMR (DMSO-$d_6$): 3.90 (3H, s), 4.01(1H, m), 4.35(2H, m), 4.58(2H, dd, J=2.48 and 14.48Hz), 6.62(1H, d, J=15.84Hz), 7.27(2H, d, J=8.92Hz), 7.68(1H, d, J=15.84Hz), 8.01(4H, m), 10.13 (1H, bs). MS (m/z): 383 $[M]^+$.

The following compounds are prepared by methods analogous to those disclosed in Examples 1-6:

**Table 1**

| Example | Structures | m/z $[MH]^+$ | Example | Structures | m/z $[MH]^+$ |
|---|---|---|---|---|---|
| 1 |  | 380 | 8 |  | 382 |
| 2 |  | 488 | 9 |  | 355 |
| 3 |  | 431 | 10 |  | 325 |
| 4 | | 474 | 11 | | 339 |

| | | | | | |
|---|---|---|---|---|---|
| |  | | |  | |
| 5 |  | 354 | 12 |  | 399 |
| 6 |  | 383 | 13 |  | 339 |
| 7 |  | 490 | | | |

## BIOLOGICAL TESTING AND ENZYME ASSAYS

### In vitro HDAC assay for determination of IC50 values

*Experimental procedure*

The assay has been carried out in 96well format and the BIOMOL fluorescent-based HDAC activity assay has been applied. Compared to the traditional HDAC assay-using radioisotope labeled substrate [1, 11, 19], this assay is more specific (p53 peptide substrate [2, 5]), easier (two steps), homogenous and sensitive (fluorescent-based). Briefly, deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore (symbol). Te fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. Currently, this assay has been successfully applied in many studies related to HDAC inhibition effects [4, 12, 14, 21]. The analytical software, Prism 3.0 has

been used to generate IC50 from a series of data. The pipetting scheme for a representative experiment is shown below: 1. add 10 ul of assay buffer into columns 2-5, 7-10, wells B,C,D,E,G11-12 (for layout 1 and 2); or all wells in rows B-G and wells H1-2 (for layout 3); 2. add 12.5 ul of 2.5X compound (in layout 1: 50 uM; layout 2: 2.5 uM; layout 3: 250 uM) into columns1, 6 and wells A11-12 (layout 1 and 2, for layout 3: wells of rowA). 12.5 ul buffer into wells F11 and 12 (layout 1 and 2, for layout 3: H3-4); 3. serials dilute 2.5 ul (5X) in orientation as indicated above; 4. discard last 2.5 ul from column5, 10 and wells E11-12 (for layout 3: from rowG); 5. add 2.5 ul of HDAC8 enzyme (0.5 U) into all wells except F11-12 (for layout 3, H1-2); 6. add 12.5 ul of 2X substrate (200 uM) into all wells; 7. incubate at RT for 2 hr with agitation; 8. add 25 ul of 2X developer into all wells and incubate for 10 mins.

*Equipment & Materials*

Equipments:

(a). Tecan Ultra Microplate detection system (Tecan Group Ltd. Switzerland)

(b). Labnet Shaker, Model 30 (National Labnet Co., Inc. Woodbridge, NJ, USA)

Materials:

(a). 96-well U-form black microplate, 650209, (Greiner Bio-One, Frickenhausen, Germany)

(b). Histone Deacetylase 8 (HDAC8) (human, recombinant), 100 U (SE-145, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(c). *Fluor de Lys*-HDAC8 Substrate, 0.5 umol (KI-178, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(d). *Fluor de Lys*™ Developer Concentrate (20x), 300 ul (KI-105, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)

(e). HDAC8 assay buffer: Tris pH7.5, 25 mM; NaCl, 137 mM; KCl, 2.7 mM; $MgCl_2$, 1 mM, BSA,

*Data analysis*

The assay is composed of duplicates for each compound. Thus, for the raw values from fluorescence reading, a mean value will be calculated using Excel formula [average (value1: value2)]; in addition, standard deviation will also be determined based on duplicates by formula [std (value1: value2)]. The Z' factor is calculated on the basis of high/ low signal with definitions: high signal= no inhibitor and low signal= no enzyme. Hence $Z'=1-3*(SD_{high}+SD_{low})/(Signal_{high}-Signal_{low})$.

The HDAC enzyme inhibition results of representative compounds are shown in Table 2.

**Table 2**

| Compound | HDAC Enzyme Activity, $IC_{50}$ (μM) |
|---|---|
| 1 | 0.119 |
| 2 | 0.355 |

| | |
|---|---|
| 3 | 1.71 |
| 4 | 0.790 |
| 5 | 0.401 |
| 6 | 0.262 |
| SAHA | 0.234 |

Cell-based proliferation assay for determination of GI50 values

The Cell proliferation assay is performed in a 96-well plate format. Cells are plated overnight and treated with compounds (in triplicates, 9-dose treatment, 4-fold dilutions from 100uM) over 96hrs. Cell growth is then determined by analysing the number of viable cells remaining following treatment of the cells. Dose response curves are plotted to determine $GI_{50}$ values for the compounds. Staurosporine treatment is used as a positive control for the experiments as staurosporine inhibits kinases and has anti-proliferative activity.

The CyQUANT cell proliferation assay is a fluorescent assay based on the measurement of cellular nucleic acid content. It contains a fluorescent nucleic acid stain, the CyQUANT GR reagent, that measures total nucleic acids as a direct indication of cell number. The assay is more rapid and convenient than conventional assays measuring metabolic activity as it does not require long incubations and cells can be frozen and stored prior to assaying. In this protocol, it is applied solely to the analysis of adherent cell lines. After treatment with compounds, detached dead cells are removed with the culture supernatant and only the viable cells remaining are quantified in the assay by fluorescence measurement at 485/535nm. In the analysis of viable cells in suspension cell lines, the CyQuant assay cannot distinguish between live and dead cells in suspension as it measures total nucleic acid content (from both live and dead cells) in solution. Thus, for analysis of suspension cell lines, methods based on measurement of metabolic activity have to be used.

The method adapted for this protocol is the Celltiter96 $Aq_{ueous}$ One Solution Cell Proliferation Assay. It is a colorimetric method for determination of cell viability based on the cleavage of an MTS tetrazolium compound into a coloured formazan product by metabolically active cells. The quantity of formazan product is directly proportional to the number of living cells in culture. The assay has to be performed immediately after compound treatment of cells. The Celltiter96 $Aq_{ueous}$ One Solution Reagent is added directly to the cells in culture and incubated for 1-4hours for colour development before recording the absorbance at 490nm.

*Equipment & Materials*

Equipment:

Vortex Mixer

Plate shaker for 96-well plates

Single and Multichannel pipettors

Cell culture Incubator

Microplate readers for reading of fluorescence(485/535nm) and absorbance (490nm)

Materials:

Appropriate cancer cell line(s) and culture media

10mM Compounds

1mM Staurosporine

96-well cell culture plates

Reagent reservoirs

For adherent cell lines: CyQUANT cell proliferation assay kit (Molecular Probes #C-7026)

For suspension cell lines: Celltiter96 $Aq_{ueous}$ One Solution Cell Proliferation Assay (Promega #G3580)

The cell activity results of representative compounds are shown in Table 3.

Table 3

| Compound | NCI H552 (μM) | Colon 205 (μM) |
|---|---|---|
| 1 | 1.31 | 0.52 |
| 2 | 1.20 | 0.43 |
| SAHA | 1.29 | 2.57 |

Histone H3 acetylation assay

A hallmark of histone deacetylase (HDAC) inhibition is the increase in the acetylation level of histones. The degree of histone acetylation can be monitored by a Western Blot approach, where specific antibodies directed against the acetylated version of histone H3 are used. Briefly, 1.5 $x10^6$ Colo 205 colon cancer cells were plated into 10 cm dishes and grown overnight in RPMI medium. Thereafter, the cells were treated with increasing amounts of HDAC inhibitory compounds by adding them into the medium (0.1, 1, 5 and 10 μM final concentration). After 12 hours of incubation the cells were harvested, lysates prepared and the Western Blot procedure carried out as described in detail below.

Western Blot approach

Specific proteins can readily be identified with the use of antibodies directed explicitly towards it. Accordingly, the more abundant protein would display a stronger signal compared to one which was present in a lower concentration.

Proteins must first be extracted from cells and quantified before equal amounts from each cell line can be separated by gel electrophoresis (SDS-PAGE).

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for Western Blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane (nitrocellulose or PVDF) in the same sequence of

separation as that on the SDS-PAGE. The membrane is then blocked with an inert protein like bovine serum albumin (BSA) or non-fat milk. This will avoid non-specific binding of the primary antibody to the un-blotted surface of the membrane.

To detect the antigen (separated protein of interest) blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane.

In order to detect the antibody which has bound, an anti-immunoglobulin antibody coupled to a reporter group such as the enzyme horse radish peroxidase is added (e.g. Goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody is bound to the protein.

PROTEIN EXTRACTION

Keep equipment cold and everything else on ice.

PROTEIN EXTRACTION BUFFER (1 ml.):

Take 200ul each of 5x Buffer, 5x NaCl, and 5x Igepal from the Sigma Mammalian Cell Lysis Kit, and make up to volume with 400ul of deionised water. Add 60 ul Protease Inhibitor Cocktail for every ml. of extraction buffer.(*Reduce Protease Inhibitor is higher volumes are used in protein extraction ~ 15ul for every ml)

Protein Extraction from cells grown in culture:

Rinse cells in ice-cold PBS and detach with trypsin. Pellet cells by centrifugation ~1300g x 5 minutes, and remove supernatant. Resuspend cell pellet in I ml. PBS and transfer into a 1.5ml. Eppendorf tube and centrifuge ~ 13000g for 10 mins. Remove supernatant and gently resuspend cells in appropriate amounts of PROTEIN EXTRACTION BUFFER (e.g ~ 50ul for small pellets and 100 – 150ul for larger pellets) Immediately freeze in $LN_2$ and allow to thaw on ice ~ 20 mins. Centrifuge at ~13000 x g for 30 minutes Remove supernatant into a new tube and either freeze in $LN_2$ and store at -80° C or keep on ice for further work.

PROTEIN QUANTIFICATION (BRADFORD METHOD)

1. Prepare protein standards of 0 – 3000 ng from a stock solution of 250ug/ml; 2. dilute all samples between 50x – 100x; 3. dilute Bio-Rad Protein Assay solution 5x and transfer 200ul into each well in a 96-well plate; 4. add 20ul of each standard or sample solution into individual wells; 5. mix for ~ 1 min.; 6. read absorbance at 595 nm.

PROTEIN SEPARATION (SDS-PAGE)

6X Sample Loading Buffer

7 ml. 0.5M Tris-HCl, 0.4% SDS, 3 ml. glycerol , 1 g SDS, 0.93 g DTT, 1.2 mg bromophenol blue. Make up volume to 10 ml with deionised water. Store in small aliquots at –20C. Add an appropriate amount of sample loading buffer to all samples. Heat to 95° C in the heating block for 5 minutes.

GEL ELECTROPHORESIS:

Set up the Xcell Sure Lock Electrophoresis gel tank according to the manufacturer's instructions. Place the pre-cast NuPage gels into the gel holders and add diluted NuPage tank buffer into the chambers.

Load the Rainbow molecular weight marker and samples into respectives wells in the gel

Connect the gel tank to the power pac and run the at 150V constant for ~ 1 hour.

PROTEIN TRANSFER – ELECTROBLOTTING

While gel is running cut PVDF membrane and filter papers to exact size of gel (8.5 x 6cm). Wet PVDF with methanol in petri-dish. Then soak PVDF in diluted NuPage transfer buffer for at least 15min. Take gel, dessemble and cut wells and stacking gel off. Make a gel sandwich (From black side down on bench):

-Sponge (make sure no bubbles)

-3x Whatman filter paper (Wet in transfer buffer)

-Gel, roll out bubbles

- PVDF, roll out bubbles

-3x Whatman filter paper (Wet in transfer buffer)

-Sponge (make sure no bubbles)

Assemble transfer apparatus. Place a small stirrer in chamber. Load transfer cassettes with the BLACK side FACING BLACK part of blotting chamber. Fill chamber with Transfer Buffer past all holes of sandwich pack. Run at 100V for 60mins or 250mA for 150 mins at 4oC. Dissemble gel sandwich.

Mark the marker on the blot according to the color and identify the protein side up.

IMMUNODETECTION (ANTIBODIES)

Reagents:

10X Tris Saline Solution (TSS) (pH 7.6)

| | |
|---|---|
| Tris 100 mM | 12.1g/L |
| NaCl 1.5M | 87.6g/L |

Adjust pH to 7.6 and store at 0-4C

Blocking Solution (1x TSS/5% milk)

| | |
|---|---|
| 10x TSS | 10ml |
| Non-fat powder milk | 5g |
| Deionised water | 90ml |

Washing Buffer (1x TSS/0.5% milk /0.1% Tween-20)

| | |
|---|---|
| 10X TSS | 100ml |
| Non-fat powder milk | 2.5g |
| Tween-20 | 1ml |
| Deionised water | 890ml |

Blocking

Transferred proteins can be visualized by staining the membrane for a few minutes with Ponceau S. Remove stain from the membrane by washing with deionised water. Place membrane into blocking solution. Block for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C.

Incubation with primary antibody

Antibodies used:

I Anti-acetylated Histone H3 (Lysin 14), UPSTATE

II Anti-Actin (SIGMA)

Protocol

Decant the blocking buffer and wash the membrane with washing buffer for another 30mins.
Add the primary antibody, diluted in washing buffer as suggested in the product description sheet.
Incubate with agitation for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C.
Incubation with secondary antibody. Decant the primary antibody. Wash the membrane 3X with washing buffer. First, for 15 mins then the subsequent 2X for 5 minutes. Decant the wash solution and add HRP-conjugated secondary antibody, diluted in wash buffer. Incubate for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C. Decant the antibody conjugate and wash for 40 minutes with agitation in wash buffer, changing the wash buffer every 10 minutes.

Substrate incubation (ECL)

Decant washing buffer and place the blot on a clean tray.Prepare enough detection substrate according to the manufacturer's specifications. Gently drop the substrate solution over the protein side of the membrane and incubate at RT between 1-5 mins. Remove the blot from the tray and place it between two pieces of write-on transparency film. Smooth over the covered blot to remove air bubbles and excess substrate and place the blot in an X-ray cassette. In the dark room lay x-ray film down over blots for 1sec - 20mins. Remove and develop film in the X-ray film processor. *All work must be carried out in the dark or only with red light.*
Reseal the cassette and replace all films into the respective box before switching on the lights.

Data analysis

Place the developed film into the UVP under white light and use the Bioimaging software to read the density of each band observed on the film.
The values are then normalised against the density of actin (or any other house-keeping protein) in the corresponding samples to obtain the expression of the protein in a particular cell line.
The results of histone deacetylase assay are shown in Table 4.

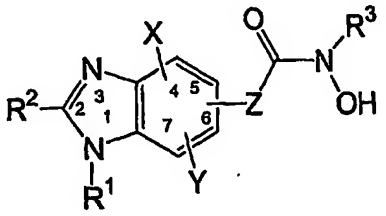
**Table 3**

| Compound | Histone 3 acetylation activity |
|---|---|
| 1 | Active |
| 2 | Active |
| SAHA | Active |

Note: The histone 3 acetylation activity of Compound 1 and 2 are similar to that of SAHA

What is claimed is:

1. A compound of the formula (I)




Formula I

wherein

- $R_1$ is selected from from the group consisting of hydrido, $C_1$ -$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- $R_2$ is H, halo, or is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy,

alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;

- $R_3$ is selected from the group consisting of H, $C_1$ -$C_6$ alkyl, acyl;
- X and Y are the same or different and are independently selected from the group consisting of hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6$ $R_7$ ;
- R4 is selected from the group consisting of C1-C4 alkyl;
- R5 is selected from the group consisting of C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and are independently selected from the group consisting of hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;
- Z is a single bond or is selected from the group consisting of CH2, -CH2CH2-, CH=CH, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C1-C4 alkyl; Z is attached to ring position 4-, or 5-, or 6-, or 7- position of Formula I;

or a pharmaceutically acceptable salt thereof.

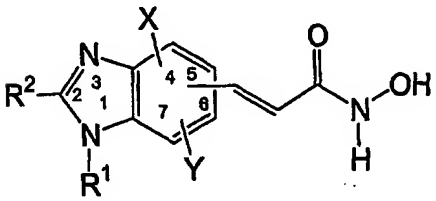
2. A compound of claim 1 wherein Z is a bond, -CH2-, -CH2CH2-, or CH=CH, and Z is attached at ring position 5- or 6-;

3. A compound of claim 1 wherein Z is –CH=CH-, and is preferably attached at ring position 5- or 6-;

4. A compound of claim 1 wherein $R_3$ = H;

5. A compound of claim 1 wherein X and Y are hydrido groups;

6. A compound of claim 1 having formula Ib:




Formula Ib

wherein

- $R_1$ is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl,

acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;

- $R_2$ is selected from the group consisting of $C_1$ –$C_{10}$ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, $C_4$ -$C_9$ heterocycloalkylalkyl, cycloalkylalkyl (e.g., cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and –NO2; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cyclalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylosulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR4, -C(O)OH, -SH, and acyl;
- X and Y are the same or different and independently selected from the group consisting of hydrido, halo, $C_1$ -$C_4$ alkyl, such as $CH_3$ and $CF_3$, $NO_2$, $C(O)R_4$, $OR_5$, $SR_5$, CN, and $NR_6R_7$;
- R4 is selected from the group consisting of C1-C4 alkyl;
- R5 is selected from the group consisting of C1-C4 alkyl, heteroalkyl, acyl;
- R6 and R7 are the same or different and independently selected from the group consisting of hydrido, C1-C6 alkyl, C4-C9 cycloalkyl, C4-C9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, and heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

7. The use of a pharmaceutical composition of claim 1 to treat proliferative diseases, including cancerous tumors.

8. The use of pharmaceutical composition of claim 6 to treat proliferative diseases, including cancerous tumors.

9. The use of a pharmaceutical composition of claim 1 to modify deacetylase activity, preferably histone deacetylase activitiy.

10. The use of a pharmaceutical composition of claim 6 to modify deacetylase activity, preferably histone deacetylase activitiy.

11. The compound of of claim 6 wherein the structure of said compound is selected from compounds, and their pharmaceutically acceptable salts, selected from the group consisting of

N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylamide;

N-Hydroxy-3-[1-(3,4,5-trimethoxybenzyl)-2-(2-phenyl-ethyl)-1H-benzimidazol-5-yl]-acrylamide;

N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-methyl-1*H*-benzimidazole-5-yl]-acrylamide;

N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-(3-hydroxy-propyl)-1*H*-benzimidazole-5-yl]-acrylamide;

N-Hydroxy-3-[1-(2-hydroxy-ethyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide;

N-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide;
N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-(2,3-hydroxy-propyl)-1*H*-benzimidazole-5-yl]-acrylamide;
N-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(2-phenyl-ethyl)-1*H*-benzimidazol-5-yl]-acrylamide;
N-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(4-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide;
N-Hydroxy-3-[1-(2-hydroxy-ethyl)-2-(4-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide;
N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(4-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide;
N-Hydroxy-3-[1-(3-pyridylmethyl)-2-(2-phenyl-ethyl)-1*H*-benzimidazol-5-yl]-acrylamide;
N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide.

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